

Alimentary Tract

Searching for wheat plants with low toxicity in celiac disease: Between direct toxicity and immunologic activation

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ABSTRACT

Background: Natural or induced variations in the noxiousness of gluten proteins for celiac disease (CD) patients are currently being investigated for their potential in breeding wheat crops with reduced toxicity. **Aims:** We evaluated the bread wheat line C173 for its effects on the *in vitro*-grown duodenal mucosa of CD patients.

Methods: *In vitro*-grown duodenal mucosa biopsies of 19 CD patients on a gluten-free diet were exposed to peptic/tryptic-digested prolamins from bread wheat line C173 lacking gliadin–glutenin subunits, analyzed for morphology, cytokine and anti-tTG antibody production, and compared with mucosa biopsies exposed to prolamins from wild-type cv. San Pastore.

Results: Duodenal mucosa biopsies exposed to prolamins from C173 and San Pastore released higher amounts of IFN- γ , IL-2, IL-10 and anti-tTG antibodies in the culture medium than untreated controls. The line C173 differed from cv. San Pastore as it did not produce negative effects on enterocyte height, suggesting that manipulating prolamins composition can affect innate immune responses of CD mucosa to wheat gluten.

Conclusions: Our data demonstrated that this gliadin-deficient wheat has a lower direct toxicity but activates an immunologic reaction of the duodenal mucosa like that of the common wheat species.

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1. Introduction

Celiac disease (CD) is the most common intestinal chronic inflammatory disease, affecting 1 in every 120–300 subjects [1–5]. CD occurs in genetically predisposed individuals and is caused by the ingestion of wheat gluten proteins which activate an autoimmune, T-cell-mediated disease. Gliadins and glutenins, collectively called prolamins, are the main components of wheat gluten. These proteins and their counterparts in cereal species such as barley, rye and triticale are all able to activate T-cell reaction and CD, but alpha-gliadins are considered the most important triggers as

their peptides are recognized much more effectively by the intestinal T-cells [6–8]. It is well known that after the elimination of gluten proteins from the diet, CD generally goes into remission, with patients relapsing when gluten is reintroduced [9]. Due to the severity of symptoms and the increased morbidity and mortality [10], CD patients must follow a life-long gluten-free diet (GFD), which eliminates both the symptoms and the risk of complications. However, a strict GFD is not easy to follow, as prolamins are to be found in cereal species that constitute the “staple foods” of the “Western diet”. For this reason, CD patients often do not fully adhere to GFD and therefore remain exposed to a high morbidity risk. Consequently, new strategies are being actively pursued to find new treatments or to eliminate noxious prolamins from cereal grains [11–14].

To date, none of the previous approaches has been successful and it seems more probable that a combination of different treat-

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ment strategies could open new therapeutic scenarios. In order to contribute to these multidisciplinary strategies, we performed an *in vitro* study on bread wheat line C173 lacking certain prolamin peptides, testing for its toxicity in CD. In detail, prolamins from line C173 and those from the commercial cultivar (cv.) San Pastore were compared for their effects on the duodenal mucosa of CD patients in terms of histological modifications and production of inflammatory-related compounds such as cytokines and antibodies against tissue-transglutaminase.

2. Patients and methods

The study was performed at the Department of Internal Medicine of the Policlinico University Hospital of Palermo between October 2005 and December 2006. Duodenal biopsy specimens were obtained from 19 volunteer CD subjects (5 males, 14 females, median age 42 years, range 18–62), who declared they had strictly adhered to GFD for at least 18 months (median 43 months) and who resulted negative for IgA anti-transglutaminase antibodies (anti-tTG). Table 1 shows the clinical characteristics of the patients.

At least six biopsy specimens were taken from the second part of the duodenum of each patient during esophago-gastro-duodenoscopy performed as described previously [15]. Three of these biopsy specimens were used for histological evaluation and three for *in vitro* culture. Only biopsy samples with no traces of villous atrophy at duodenal histology evaluation were used for these studies.

All participating patients provided written informed consent and the study was approved by the Ethics Committee of the University Hospital of Palermo.

2.1. Prolamin extraction and digestion

Bread wheat (*Triticum aestivum*) cv. San Pastore and line C173 were compared in this study. cv. San Pastore is a very old bread wheat variety grown in thousands of hectares in Italy during the decades 1950–1970. It shows a wild-type prolamin pattern, with a strong toxic effect in CD. On the other hand, bread wheat line C173 is an experimental genotype obtained from breeding two mutant plants with the spontaneous deletion of genes encoding for several gliadin and glutenin proteins [16]. In particular, line C173 simultaneously lacks 6–8 α/β -gliadins encoded by the *Gli-A2* locus and several γ - and ω -gliadins encoded by the complex *Gli-D1/Glu-D3* locus, which also codes for a few low molecular weight glutenin subunits [16]. The electrophoresis pattern of gliadins from line C173 has been shown previously [17].

Kernels (100 g) of cv. San Pastore and line C173 were milled and mixed with 400 ml (w/v) of 0.5 M NaCl. The suspension was stirred at 4 °C for 1 h and then centrifuged at 2000 $\times$ g for 15 min at 4 °C. Proteins in the supernatant (albumin and globulin) were eliminated. This operation was repeated twice. The pellet was resuspended in 300 ml of 70% (w/v) ethanol, extracted for 1 h at room temperature under stirring, and centrifuged at 2000 $\times$ g for 20 min at 20 °C. The supernatant containing prolamins was frozen and freeze-dried. Prolamins were submitted to peptic-tryptic (PT) sequential digestion as described by De Ritis et al. [18]. At the end of the procedure, the PT digests were heated for 30 min at 100 °C, lyophilized and stored at –20 °C.

2.2. Ex vivo organ cultures

Cultures were performed according to the method of Fais et al. [19]. Briefly, biopsies were orientated villous side up on a nylon mesh gauze (Millipore) and placed in the central well of a 2-ml tissue culture dish containing RPMI 1640 supplemented with foetal calf serum and 1% antibiotic-antimycotic solution. From each

patient, one mucosa biopsy was cultured in a dish with RPMI 1640 (control), one was cultured after the addition of 5 mg/ml of the prolamin PT digest from cv. San Pastore and one was cultured after the addition of 5 mg/ml of the PT digest from line C173. The culture dishes were placed in an organ culture chamber, gassed with a mixture of 95% O₂ and 5% CO₂, and incubated at 37 °C for 24 h. At the end of the culture period, supernatant fluid from the cultured specimens was collected and stored at –70 °C until used, and the biopsies were embedded in OCT compound and snap-frozen for cryosectioning for immunostaining.

2.3. Cytokines assay in the culture medium

Supernatants were stored at –80 °C until assay. Interleukin (IL)-2, IL-10 and interferon (IFN)- γ were determined in the supernatants by the Luminex 100 System at the Cytokine Core facility of the University of Maryland, Baltimore. For detailed information on the Luminex 100 system, see the cytokine core facility website (www.cytokines.com). The IFN- γ values have a range of 12.3–9000 pg/ml; both IL-10 and IL-2 have a range of 6.9–5000 pg/ml.

2.4. Immunohistochemistry study

The snap-frozen biopsy samples were sent to the laboratory of the University of Brescia for the immunohistochemistry study. Cryostat sections (5 μ m thickness) were used and the study was performed according to a standard technique for anti-CD3 monoclonal antibody (Beckton-Dickinson, USA dilution 1:20) to better identify the intra-epithelial lymphocytes (IEL). All slides were coded and analyzed in blind by an expert pathologist (VV). Duodenal histological lesions were classified according to two criteria: (1) the standard classification proposed by Marsh [20] and subsequently modified by Oberhuber [21] (M–O), and (2) the recently proposed simplified classification for CD [22,23]. Recent criteria have established the upper normal limit for IEL as 25 IEL/100 epithelial cells [24]. We considered a mucosa with a villous/crypt ratio of 3:1 (plus a normal IEL count) as normal, whereas a decrease in the villous/crypt ratio led to the following definition of atrophy: 2:1 ratio = mild atrophy; 1:1 ratio = moderate atrophy; no villi (flat mucosa) = total atrophy.

Enterocyte height after culture was measured according to Howdle et al. [25]. The measured enterocytes were on the lateral upper third of the villi and at least 20 enterocytes were measured in each section and the mean was then calculated.

The pathologist was unaware of the culture conditions.

2.5. Anti-transglutaminase IgA antibody (anti-tTG) assay in the culture medium

Supernatant fluid specimens were sent to the laboratory of Eurospital (Trieste, Italy) for anti-tTG assay. Samples were assayed according to the method previously described [26]. In brief, recombinant h-tTG antigen diluted in phosphate-buffered saline was used to coat the wells. The purity of the recombinant protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The culture medium was diluted 1:5. The conjugate was diluted to obtain reliable absorbances, which were read in a microplate reader at 450 nm. Anti-tTG values in the supernatants were expressed as absorbance. Reference values were taken as <300, representing a value <2 SD above the mean of 200 healthy individuals. The intra-assay CV for the IgA h-tTG autoantibody ELISA on culture medium was 3.2%, and the inter-assay CV was 5.4% [26].

Previous studies in which IgA anti-tTG antibodies were first assayed on fresh medium and then six months later after storage at

Table 1
Patients characteristics.

Case	Sex	Age at the time of the study	Duodenal histology at diagnosis	Serum EmA at diagnosis	Duodenal histology at the time of the study	Serum anti-tTG at the time of the study
1	F	42	Marsh 3A	Positive	Marsh 1	Negative
2	M	18	Marsh 3B	Positive	Marsh 2	Negative
3	F	62	Marsh 3B	Positive	Marsh 2	Negative
4	F	60	Marsh 3A	Positive	Marsh 1	Negative
5	F	20	Marsh 3C	Positive	Marsh 1	Negative
6	M	55	Marsh 3B	Positive	Marsh 1	Negative
7	F	24	Marsh 3B	Positive	Marsh 0	Negative
8	F	49	Marsh 3C	Positive	Marsh 1	Negative
9	M	21	Marsh 3A	Positive	Marsh 1	Negative
10	F	52	Marsh 3C	Positive	Marsh 1	Negative
11	F	26	Marsh 3A	Positive	Marsh 1	Negative
12	M	60	Marsh 3C	Positive	Marsh 0	Negative
13	F	28	Marsh 3C	Positive	Marsh 0	Negative
14	M	50	Marsh 3C	Positive	Marsh 1	Negative
15	F	22	Marsh 3B	Positive	Marsh 2	Negative
16	F	29	Marsh 3B	Positive	Marsh 1	Negative
17	F	51	Marsh 3B	Positive	Marsh 0	Negative
18	F	33	Marsh 3C	Positive	Marsh 0	Negative
19	F	58	Marsh 3C	Positive	Marsh 0	Negative

Abbreviations: EmA: anti-endomysium antibodies and anti-tTG: anti-transglutaminase antibodies.

–80 °C, had shown that storage did not alter the results obtained, as anti-tTG results were identical [26]. IgA anti-tTG assays on the culture media were performed by personnel unaware of the clinical and laboratory data of the patients.

2.6. Statistical analysis

Mean values $\pm$ S.D. of enterocyte height, IEL count, cytokines and anti-tTG values were calculated. The T-test for paired data was used to compare the results. Bonferroni's adjustment of the probability estimation was performed to minimize the bias due to the low number of samples evaluated. *P* values <0.05 were considered significant.

3. Results

3.1. Cytokines assay in the culture medium

Fig. 1 shows the mean values ($\pm$ S.D.) of IFN- γ in the culture medium of the duodenal biopsies exposed to the PT digest of

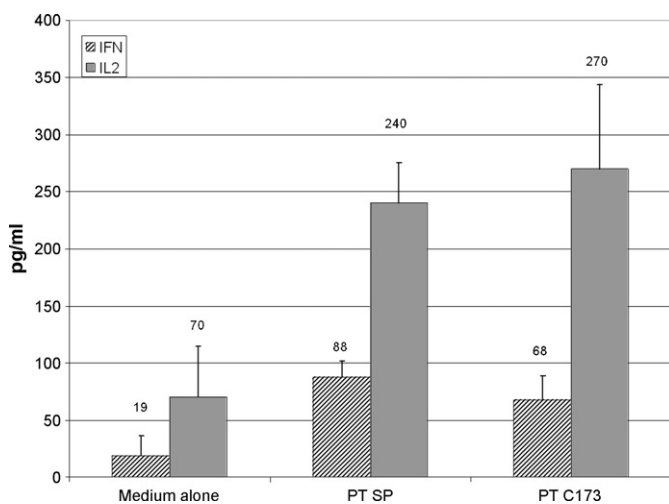


Fig. 1. Mean values ($\pm$ S.D.) of interferon-gamma and interleukin-2 in the culture medium of intestinal biopsies cultured with medium alone, or with the addition of peptic-tryptic (PT) digest of the common cultivar “San Pastore” (SP), or with the addition of PT digest of the gliadin-deficient species “C173”.

prolamins from cv. San Pastore or line C173, compared to those of untreated controls. The addition of PT digests determined a significant increase in cytokines in the culture medium in both cases. In particular, IFN- γ concentration was higher in the culture media containing prolamins from cv. San Pastore ($t = 3.746$; $P = 0.005$) and from line C173 ($t = 3.334$; $P = 0.01$) than in the base (control) medium. No significant difference in IFN- γ production was observed in the biopsy samples stimulated with prolamins either from cv. San Pastore or from line C173. Similarly, IL-2 production (Fig. 1) was higher in the culture media of biopsies exposed to PT digests from cv. San Pastore ($t = 2.823$; $P = 0.03$) and line C173 ($t = 3.163$; $P = 0.017$) than in the control media. No significant difference in IL-2 production was observed between the mucosa samples stimulated with prolamins from cv. San Pastore and those exposed to prolamins from the mutant line C173. Finally, IL-10 concentrations in the culture media of the treated biopsies (180 ± 105 pg/ml and 190 ± 80 pg/ml for cv. San Pastore and line C173, respectively) were higher than those of the untreated controls (130 ± 100 pg/ml). This increase was statistically significant ($t = 2.235$; $P = 0.04$) for the mucosa biopsies exposed to prolamins from line C173.

3.2. Immunohistochemistry study

None of the duodenal mucosa samples included in the study showed villous atrophy before the *in vitro* culture. After 24 h culture with the medium alone, all the non-stimulated samples showed type 0 or type 1–2 lesions, according to the Marsh–Oberhuber classification [20,21]. Concordant data were obtained with the Corazza classification [22]: no samples showed villous atrophy (type A). No significant villous damage was observed in the mucosa samples after culture with the addition of PT digests of the cv. “San Pastore” or the addition of the gliadin-deficient wheat species “C173”.

Mean enterocyte height ($\pm$ S.D.) was 30.1 ± 2.5 μ m before culture and 27.7 ± 2.1 after culture in the control medium ($P < 0.05$). Fig. 2 shows the mean enterocyte height ($\pm$ S.D.) of the samples cultured in the medium alone compared with the samples cultured after the addition of the two PT digests. No statistical difference was observed between the duodenal samples cultured after adding the “C173” PT, whereas mean enterocyte heights of the samples cultured after adding the cv. “San Pastore” were significantly lower than those of the samples cultured in the medium alone ($P < 0.01$).

Mean IELs count ($\pm$ S.D.) evaluated with anti-CD3 monoclonal antibody showed a trend towards a higher IEL number in the stim-

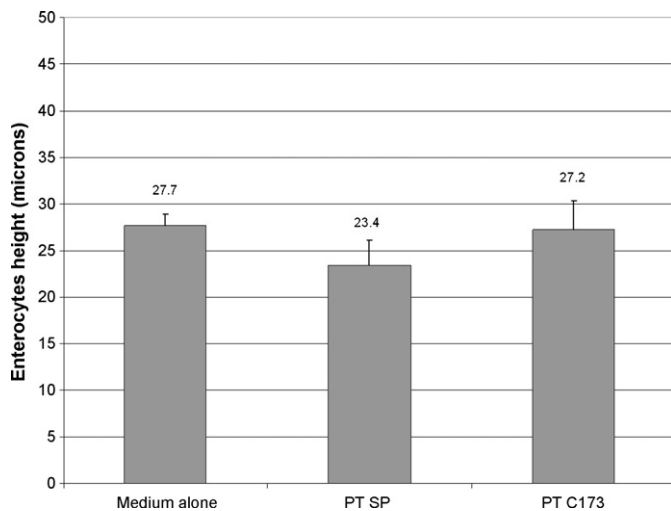


Fig. 2. Mean values ($\pm$ S.D.) of the enterocyte height of the intestinal biopsies after 24-h culture with medium alone, or with the addition of peptic–tryptic (PT) digest of the common cultivar “San Pastore” (SP), or with the addition of PT digest of the gliadin-deficient species “C173”.

ulated cultures (Fig. 3), although there was no significant difference between the samples cultured in the medium alone (24.3 ± 7.5) and in those cultured after the addition of the PT digest of the cv. “San Pastore” (28.1 ± 7.7) or the PT digest of the gliadin-deficient wheat species “C173” (27.6 ± 9.2).

3.3. Anti-transglutaminase IgA antibody (anti-tTG) assay in the culture medium

Table 2 shows the results of the anti-tTG assay in the supernatants of the culture systems. Mean optical density values of the samples cultured with the addition of the PT digest of cv. “San Pastore” and of the samples cultured with the addition of the gliadin-deficient species “C173” were significantly higher than those of the samples cultured with the medium alone ($P < 0.01$ for both). No statistical difference was observed in anti-tTG values between the samples cultured with the addition of the two different wheat species. No sample was positive ($OD > 300$) after culture in the medium alone, whereas ten subjects were positive with the addition of the PT digest of wheat “San Pastore” and nine with the addition of wheat “C173”. A significantly shorter duration of GFD was observed in the patients whose samples were positive after PT addition than in those negatives for anti-tTG *in vitro* production.

4. Discussion

CD is a worldwide problem and is estimated to affect approximately 1% of the general population in Europe and North and South America, thus constituting an important public health problem. CD treatment is based on the avoidance of foods containing wheat, rye and barley proteins. However, total avoidance is extremely difficult both due to the presence of “occult” traces of these proteins in many foods and mainly to the psychological difficulty of hav-

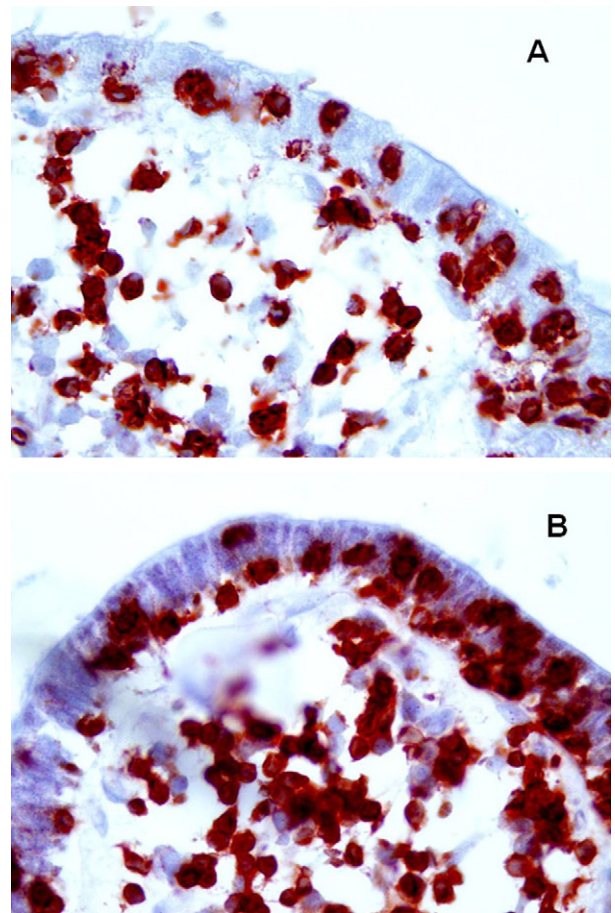


Fig. 3. Pathological increase in T lymphocytes $CD3 \times 100$ in a biopsy sample cultured with the addition of the peptic–tryptic (PT) digest of the common cultivar “San Pastore” (panel A), or with the addition of PT digest of the gliadin-deficient species “C173” (panel B).

ing to strictly adhere to the elimination diet. For this reason the search for a “non-toxic wheat” which could permit CD patients to eat widely-consumed foods such as bread or pasta is continuing [27–29].

Some studies with similar aim have been performed with the availability of T-cell lines and clones to test antigenic peptides and to predict T-cell stimulatory sequences. However, the studies using T-cell clones do not necessarily reflect the small intestine *in vivo*, where a heterogeneous population of cells interact in response to the gluten. On the contrary, the organ culture system, using whole tissue, may mirror the *in vivo* situation more accurately as demonstrated in previous studies [30].

An ideal non-toxic wheat should lack the peptides which can stimulate the innate immunity response and bind DQ2 or DQ8 and consequently activate the intestinal T lymphocytes.

cv. San Pastore and mutant line C173 differ from each other for the presence/absence of the *Gli-A2* and *Gli-D1/Glu-D3* loci. In this study, the two wheat genotypes were compared for the noxious

Table 2

Results of the anti-tTG assay in the supernatant of the mucosa specimens cultured with medium alone, with the addition of the peptic–tryptic (PT) digest of the common wheat “San Pastore”, or with the addition of the PT digest of the gliadin-deficient species “C173”. Mean values $\pm$ S.D. are expressed as optical density (OD).

	Medium alone	With addition of PT digest of wheat “San Pastore”	With addition of PT digest of wheat “C173”
Mean $\pm$ S.D. (OD)	220 $\pm$ 155	354 $\pm$ 254	339 $\pm$ 290
Number of samples with OD > 300	0/19	10/19	9/19

Note: Reference values were taken as <300 , representing a value <2 S.D. above the mean of 200 healthy individuals. Anti-tTG: anti-transglutaminase and PT: peptic–tryptic.

effects of their PT-digested prolamins on the duodenal mucosa of 19 CD patients on a GFD. The Gli-A2[−]/Gli-D1[−] seeds used have been produced previously [16] and are deficient in a series of alpha, beta, gamma and omega gliadins, including the alpha-gliadin described by Kasarda et al. [31] and the 31–43 sequence determining histology damage after challenge [32,33]. In a previous *in vitro* study these Gli-A2[−]/Gli-D1[−] seeds did not affect enterocyte height [17], considered to be a marker of wheat toxicity in CD. Furthermore, residue 31–43 (LGQQQPFPPQPPY) of A-gliadin was found to be very active in triggering mucosa lesions and enterocyte apoptosis, as well as in up-regulating genes coding for interleukin (IL)-15, cyclooxygenase-2 (COX-2) or cell activation markers CD25 and CD83 on macrophages, monocytes and dendritic cells [34]. Histology data from the present study confirmed those previously obtained since no difference in enterocyte height was seen between the specimens cultured with the medium alone and those cultured with the addition of the PT digest of the “C173” (Gli-A2[−]/Gli-D1[−]). On the contrary, the addition to the culture medium of PT digest from “San Pastore” caused a significant reduction in enterocyte height. This lack of toxicity of the Gli-A2[−]/Gli-D1[−] seeds could be due to the absence of the 31–43 peptide which seems to have a pivotal role in the non-“immunogenic” intestinal damage in CD patients. The 31–43 peptide, in fact, by means of a non-T-cell mediated effect, determines the actin and cytoskeleton rearrangements [35] which are considered an early phase of the intestinal mucosa damage in CD [36].

However, evaluation of the cytokine pattern in the culture medium revealed that the “C173” – Gli-A2[−]/Gli-D1[−] – played an identical immunogenic role to that of the cv. “San Pastore”. In fact, the concentration of γ -IFN in the culture medium of the mucosa samples cultured with the addition of PT digest of “C173” was not different from that of the samples cultured with the addition of PT digest of cv. “San Pastore”. Both these *in vitro* stimulations determined a significantly higher γ -IFN concentration increase than the non-stimulated samples. γ -IFN dominates the pattern of cytokines produced in CD intestinal mucosa after gliadin activation [37]. Also in the case of another pro-inflammatory cytokine – IL-2 – there was similarly no difference in concentration in the culture medium between the mucosa samples cultured with the PT digest of cv. “C173” and those cultured with the addition of PT digest of cv. “San Pastore”. In addition, IL-10 concentration was higher in the *in vitro*-stimulated samples than in the non-stimulated controls, although this difference was significant only for the samples cultured with the addition of PT digest of the gliadin-deficient wheat “C173”. IL-10 is known to play an important anti-inflammatory role and its increase in CD intestinal mucosa after gliadin stimulation has been previously described [38,39]. Thus our *in vitro* culture experiments gave results in agreement with those studies and with the hypothesis of new therapeutic strategies based on IL-10 promoted tolerance [40].

These results are in keeping with the presence in both cv. San Pastore and cv. C173 of the 33-mer activating peptide, mainly present in the alpha-gliadins, containing a cluster of epitopes not digested by the intraluminal and brush border enzymes, highly recognized by the immune cells [41].

Apart from cytokine activation, our data showed that the gliadin-deficient cv. C173 determined *in vitro* CD-specific antibody production. Previous studies have shown EmA [42,43] and/or anti-tTG production [26] after gliadin or 31–43 peptide *in vitro* stimulation.

The evidence of anti-tTG production after stimulation with the PT digest of “C173”, a wheat lacking the 31–43 peptide, demonstrates that this peptide is not the sole prolamins sequence able to elicit CD-specific antibody production. Furthermore, our data confirm that the *in vitro* stimulation of the duodenal mucosa of CD patients on GFD is not able to determine antibody production in all

samples, but only in a percentage of cases [44] and this is related to the duration of GFD [44,45].

Finally, it is noteworthy that the C173 wheat has good production yields and baking qualities and, consequently its widespread usage can be recommended. This could result in a “pressure reduction” of toxic gluten exposure on the general population. Consequently, this could prevent a percentage of new CD cases as there is evidence that the amount of gluten exposure is related to population incidence.

In conclusion, we are living in an era of exciting research aimed at finding an alternative to the gluten-free diet for CD patients [46]. Promising results have come from studies on the use of lactobacilli [47,48], on intestinal permeability [49] and on immunology [35,39,40,50]. The present data demonstrate that manipulation of prolamins composition can result in a wheat flour with reduced toxicity for CD enterocytes, suggesting that the association of the experimental approaches quoted above with the breeding of less toxic wheat cultivars could contribute to reach the goal in a near future.

Conflict of interest statement

None declared.

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